Supplementary Material on the Methodology Part X

Microchemistry analysis of European hake otoliths

We used Laser ablation ICPMS to analyze the elemental composition of different parts of the otolith core and edge of the European hake from the Atlantic and the Mediterranean. Otolith microchemistry is used as a possible tool for defining the stocks of European hake, following habitat use and to assess the plausible difference between individuals' spawning/nursery areas.

Data analysis

The element concentration in otoliths core and edge were determined by LA-ICPMS. Four chemical elements were measured for each otolith. Values of elements that were consistently measurable above the LOD were retained for the statistical analysis.

For the core and the edge otolith element composition, we run the same series of analyses; Firstly, we conducted a MANOVA and an univariate analysis of variance (ANOVA) to assess whether individual element:Ca ratios differed significantly among regions. Post-hoc Fisher LSD tests were used to determine the nature of significant differences among areas.

A forward stepwise linear discriminant analysis was built for core and edge data to classify each individual to one of the sites from which they were collected. Classification accuracies for each species and environment were evaluated through the percentage of correctly classified individuals using Jackknifed classification. It should be noted that Jackknife classifications are particularly sensitive to low sample size, indeed the confidence in the degree of "true" group separation will be low. For this reason, we considered only the geographical subarea (GSA) as groups (>30 individuals per group) for all the analysis.

Results

laser ablation of the otolith hake displayed different levels of Sr/Ca, Mn/Ca and Ba/ca ratios (higher in the core) and Mg/Ca between the core and the edge of otoliths sampled from the different areas. the main pattern of element concentration was partially different between core end edge (Figure 1).

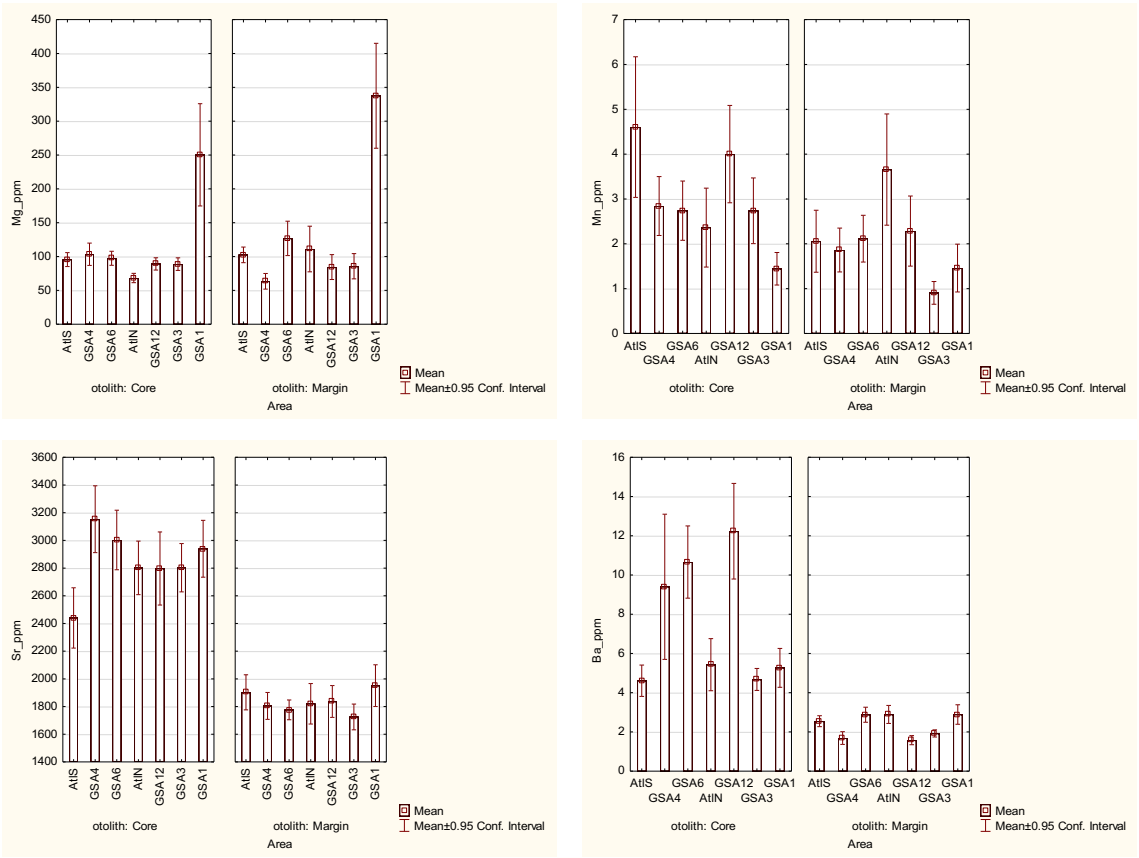

Figure 1. Comparison of individual element:Ca in otolith core and edge in European hake collected from GSAs 1, 3, 4, 6 and 12, AtlN (Atlantic North) and AtlS (Atlantic South) for the following elements Mg (a), Mn (b), Sr (c) and Ba (d)

Element core

The structure of four element Mg:Ca, Sr:Ca, Ba:Ca and Mn:Ca from otolith core was analyzed. By applying MANOVA significant difference was found among the collection locations ($F = 11.85$; p value = 0.000**). Microchemistry analysis of the hake otolith core was found to be effective in detecting differences in elemental concentration across the different areas using ANOVA (Table 1).

Table 1. Results of univariate ANOVA and Post-hoc Fisher LSD tests comparing individual element ratios in otolith cores of European hake collected from GSAs 1, 3, 4, 6 and 12 and AtlN and AtlS

Source	df	F	P	Post-hoc Fisher LSD test differences by GSA
Mg:Ca Area	6	18.17	<0.000**	GSA1> GSA4*, GSA1>GSA12*, GSA1> GSA3* GSA1>GSA6* GSA1>AtlS* GSA1>AtlN* AtlS>AtlN* GSA3 >AtlN* GSA4 >AtlN* GSA12 >AtlN*
Mn:Ca Area	6	7.18	<0.000**	GSA4>GSA1*, GSA12>GSA1*, GSA3>GSA1* GSA6>GSA1* AtlN>GSA1* AtlS>GSA1* AtlS>AtlN* GSA12 >AtlN*

Sr:Ca Area	6	5.3	<0.000**	AtlS<GSA1* AtlS<GSA3* AtlS<GSA4* AtlS<GSA6* AtlS<GSA12* AtlS<AtlN* GSA4>GSA12* GSA4>GSA3* GSA4>AtlN*
Ba:Ca Area	6	15.44	<0.000**	GSA12>GSA4*, GSA12>GSA3*, GSA12>GSA1*, GSA12>AtlN*, GSA12>AtlS*, GSA3<GSA12* GSA3<GSA4* GSA3<GSA6* AtlS<GSA4* AtlS<GSA6* AtlS<GSA1* GSA6>GSA4* GSA4>AtlN* GSA6>AtlN*

ANOVA indicated significant differences in Mg/Ca ratio of otolith cores among the different areas with the highest Mg:Ca concentration observed in the core was found in GSA 1 and the lowest concentration in Atlantic north. Post-hoc Fisher LSD test showed no difference in Mg:Ca between GSAs 4, 3, 6 and 12 and AtlS areas.

For Manganese, the highest Mn:Ca concentration was found in AtlS followed by GSA 12 and the lowest concentration in GSA 1 which differed significantly with all the other areas.

Sr:Ca ratio showed the highest concentration at GSA 4 and the lowest one in AtlS which differed significantly with all the other areas.

Barium showed significant differences in samples among collection locations. The concentration of Ba:Ca in the otolith core was highest at GSA 12 and lowest at GSA 3.

Element edge

Otolith multi-element fingerprints of the hake otolith edge showed high significant differences with MANOVA (F=10.94, p value= 0.000**). Analysis of the microchemistry of hake otolith edge by ANOVA was also find to be effective in detecting differences in element:ca concentration between areas (Table 2).

For Magnesium, significant differences were observed between the areas with the highest Mg concentration was observed in GSA 1 and the lowest in GSA 4.

For Manganese, significant difference was observed between the different areas. the highest Mn:Ca concentration was found in AtlN followed by GSA 12 and the lowest concentration was observed in GSA 3.

For strontium there is no significant differences in hake otolith edge among the different areas in Mediterranean and atlantic.

Barium in the hake otolith edge was the highest in AtlN and the lowest in the GSA 12 Post-hoc Fisher LSD test showed no differences in concentration of Ba between GSAs 12 and 4.

Table 2. Results of univariate ANOVA and Post-hoc Fisher LSD tests comparing individual element ratio in otolith edges of European hake collected from GSAs 1, 3, 4, 6 and 12 and AtlN and AtlS

Source	df	F	P	Post-hoc Fisher LSD test differences by areas
Mg:Ca Area	6	18.66	<0.000**	GSA1>GSA4*, GSA1>GSA12*, GSA1>GSA3*, GSA1>GSA6*, GSA1>AtlN* GSA1>AtlS* GSA12>GSA4*, GSA3>GSA4*, GSA6>GSA4*, AtlS>GSA4*, AtlN>GSA4*, AtlS>GSA13* AtlS>GSA3*, GSA6>GSA12*, GSA6>GSA3*, GSA6>GSA1*, AtlS>GSA12*
Mn:Ca Area	6	5.14	<0.000**	AtlN>AtlS* AtlN>GSA4*, AtlN>GSA3*, AtlN>GSA1*, AtlS>GSA3*, GSA4>GSA3*, GSA12>GSA3*GSA1>GSA3*, GSA6>GSA3* GSA6>GSA1*
Sr:Ca Area	6	2.544	0.10	No significant diffrences
Ba:Ca Area	6	16.79	<0.000**	AtlS>GSA4* AtlS>GSA12* GSA4<GSA6*, GSA4<AtlN*, GSA4<GSA3*, GSA4<GSA1*, GSA6>GSA12* GSA6>GSA3* GSA12<AtlN* GSA12<AtlS* GSA3<AtlN* GSA12<GSA3*, GSA1>GSA12*, GSA3<AtlS*, GSA1>GSA4*, GSA3<GSA1*

Analysis of European hake otolith microchemistry in the Mediterranean

Core

A forward stepwise linear discriminant model analysis was built with Mn and Mg contributing to the first discriminatory axis which explained 64% of the variation and Ba forming the second axis which explained 28% of the variation. The plot indicates some overlap especially among samples from GSAs 4 and 3, and between GSAs 12 and 6. The samples from GSA 1 was more separated with a tendency of higher Mg:Ca and lower Mn:Ca (Figure 2)

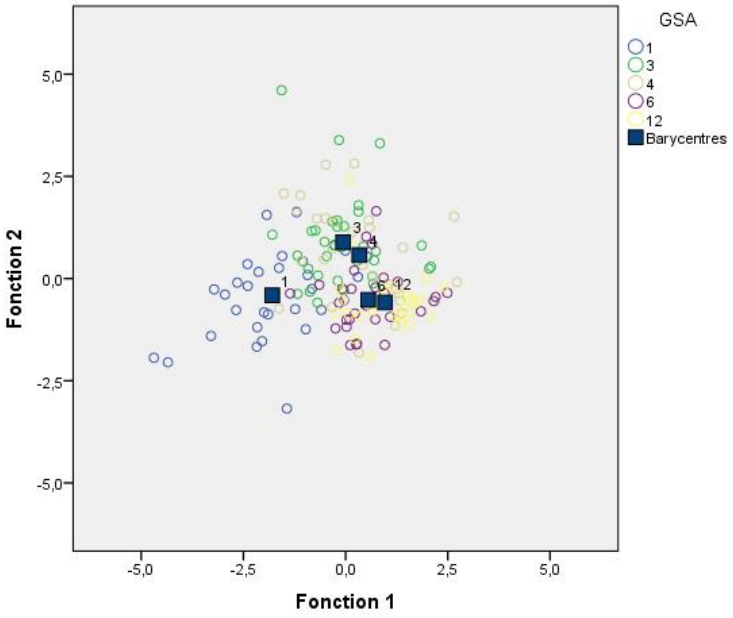

Figure 2. Scatter plot of scores obtained by DA of multi-element chemistry of otolith cores of European hake collected from GSAs 1, 3, 4, 6 and 12

The jackknifed classification procedure involving all the GSAs was accurate in assigning individual from GSA 1, GSA 3 and with lesser accuracy for GSA 3. but performed poorly for GSAs 6 and 4. The classification errors were associated with a lack of clear distinction between GSAs 4 and 3 and between GSAs 12 and 6 (Table 3).

Table 3. Results of jackknife classification of individual based on multi-element chemistry of otolith cores

	Area classified to (% sample)				
Jacknife classification	GSA1	GSA 3	GSA4	GSA6	GSA12
GSA1	**73.3**	20	3.4	3.3	0
GSA 3	15.2	**60.6**	9.1	3	12.1
GSA 4	3.4	37.9	**27.6**	17.2	13.8
GSA6	6.5	6.5	12.9	**35.5**	38.7
GSA12	0	6.7	3.3	46.7	**43.3**

Edge

A forward stepwise linear discriminant model analysis was built with Mg and Ba contributing to the first discriminatory axis which explained 69.7% of the variation and Sr, Mn forming the second axis which explained 19.9% of the variation. The plot indicates some overlap especially among samples from GSAs 12 and 4, also an overlap of GSA 6 was observed with GSAs 1 and 3 (Figure 3).

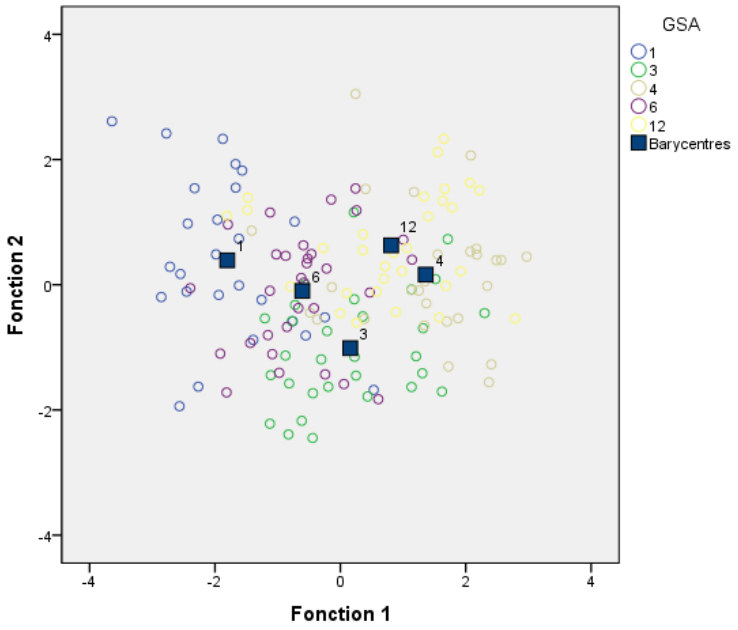

Figure 3. Scatter plot of scores obtained by DA of multi-element chemistry of otolith edges of European hake collected from GSAs 1, 3, 4, 6 and 12

The jackknifed classification procedure with all GSAs included was moderately accurate in assigning individuals to their collection GSA based on Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca ratios of their otolith edge (Table 4)

Table 4. Results of jackknife classification of individual based on multi-element chemistry of otolith edges

	Area classified to (% sample)				
Jacknife classification	GSA1	GSA 3	GSA4	GSA6	GSA12
GSA1	**57.7**	7.7	0	34.6	0
GSA 3	3.3	**66.7**	13.3	10	6.7
GSA 4	0	3.7	**63**	14.8	18.5
GSA6	9.7	16.1	6.5	**58.1**	9.7
GSA12	10	10	33.3	3.3	**43.3**

Classification accuracy was highest for the GSA 3 at 66.7% followed by GSA 4 at 63% and then GSA 6 at 58.1%. Classifications errors of GSA 3 individuals were mostly related to individuals being misclassified in GSA 4, and for GSA 4 classification errors were mostly due to misclassifications in GSAs 12 and 6.

Analysis of European hake otolith microchemistry in the Mediterranean and Atlantic

Core

A forward stepwise linear discriminant model analysis was built for core and edge otolith data to classify hake from Atlantic and Mediterranean. For the core, classification accuracy was highest for GSA 1 and relatively low for the other locations. The plot indicates some overlap especially among samples from GSAs 12, 6 and 4, and among samples from GSA 3 with Atlantic North and south. The samples from GSA 1 were clearly separated from those of the other locations (Figure 4).

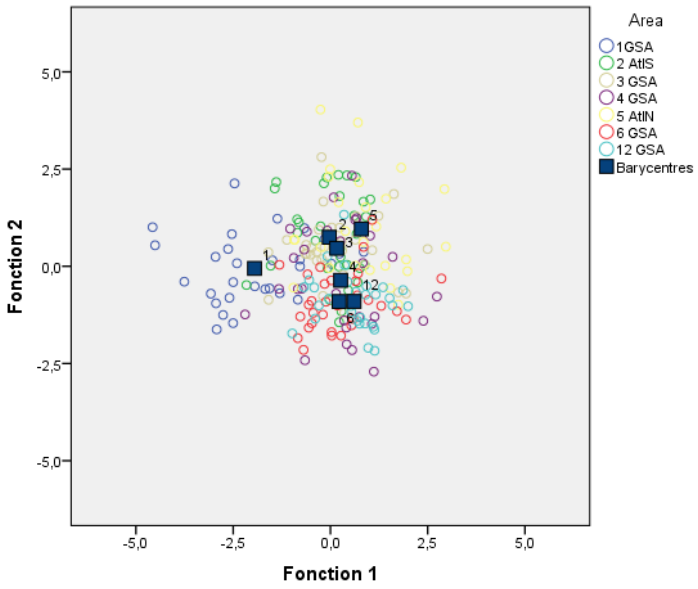

Figure 4. Scatter plot of scores obtained by DA of multi-element chemistry of otolith cores of European hake collected from GSAs 1, 3, 4, 6 and 12 and AtlN and AtlS

Edge

For the edge, classification accuracy was highest for GSA 12 samples at 80% followed by GSA 3 at 63%. The canonical variate plot indicated some overlap among individual sampled from Atlantic north, south and GSAs 6 and 1 and an overlap of individual from GSA 4 with those from GSA 12 (Figure 5).

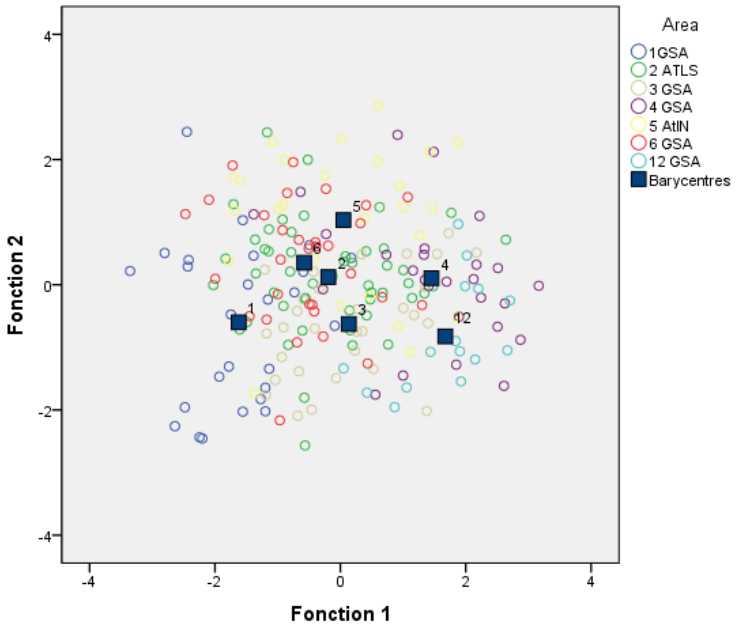

Figure 5. Scatter plot of scores obtained by DA of multi-element chemistry of otolith edges of European hake collected from GSAs 1, 3, 4, 6 and 12 and AtlN and AtlS

Discussion and conclusions

Merlccius merluccius is an important commercial demersal species in the Mediterranean Sea and in the Atlantic. It can reach depths from 25 to 1000m, although the most abundant catches are observed between 100 and 400m depth, while the eggs and larvae appear in the pelagic area at depths ranging from 50 to 150m (Orsi Relini *et al*., 2002). Little is known about the stock structure of the hake. Multivariate comparison of chemical element concentrations between core and edge zones of the otolith was applied to discriminate the stocks between the different fishing locations.

Laser ablation ICPMS is used to analyse the elemental composition of different parts of the otolith. The core represents the first weeks of age when the eggs and larvae of the hake were pelagic, and the edge represents the signal in recently deposited material. The multi-element signals may reflect populations that live and grow in a discrete area or follow set migration routes also can characterize the nursery areas of the species, clarify the connectivity between nursery and recruitment and therefore could be useful for discriminating between stocks.

The four elements Ba:Ca, Mg:Ca, Mn:Ca and Sr:Ca demonstrated variation among the different fishing locations.

For Ba there is a great evidence across multiple species that incorporation into otolith is driven principally by ambient concentration (Bath *et al*.; 2000; Hamer *et al*., 2003, 2006). Differences in otolith Ba can therefore be assumed to be indicative of variation in the ambient Ba level that the fish was exposed to. This element is often associated with high primary productivity and exhibits a nutrient-type profile, with surface depletion and enrichment at depth. So the different level of otolith Ba between the core (low value) and the edge (high value) among the different

areas could be related to shifts in the spatial and or depth distribution between larvae (pelagic region) and adult stage (deeper waters). Among the areas, the levels of Ba in the otolith core of specimens from GSAs 12 and 6 were comparable and practically the double of those from GSAs 1 and 3 and AtlN and AtlS.

Otolith Mg is not affected by either temperature and/or salinity but is correlated with otolith precipitation and somatic growth rate (Artetxe-Arrate *et al*., 2019; Bath, Martin and Thorrold, 2005). GSA 1 samples had the highest concentration of Mg and differed significantly from the other areas in the edge as well as and in the core.

Mn incorporation into the otolith is physiologically regulated and sensitive to growth effects. According to Ruttenberg (2006), high level of Mn near the otolith primordium were related to maternal transfer. The patterns of otolith Mn variation as well as concentration were similar for both the otolith core and edge among GSAs 4, 6 and 12.

Sr incorporation into otoliths has been shown to be correlated with ambient concentration, and thus appear to be reliable 'geographical marker' (Zimmerman, 2005). The concentrations of Sr reflect salinity changes and deposition of Sr in the otoliths might diminish with age (Weatherley and Gill, 1987). The different level of otolith Sr between the core and the edge among the different GSAs characterized by high level of Sr in the core compared to the otolith edge could be related to a reduction of fish metabolism with age and or a shift in a spatial and or depth distribution between larvae and adult life stage that inhabiting different salinity and temperature regimes indeed this species migrates to deeper waters and its mobility increased along the slope with age. The level of Sr in the otolith core differed significantly among the areas. The lowest concentration of Sr was observed in AtlS and AtlN which may be due to lower salinity in these areas, given that Sr concentration in the water is positively correlated with ambient salinity. In the edge there is no significant differences in the Sr;Ca ratio between the different areas however the concentration of Sr in GSAs 4 and 6 and AtlN and GSA12 are the most comparable in Sr concentration.

Significant differences found in multi-element of otolith composition by ANOVA and MANOVA could indicate the presence of some potential hake stock structure.

The canonical variate plots of the otolith core chemistry indicated relative tighter clustering of the samples from GSA 1 compared to the other GSAs. This clustering was responsible for the relatively high classification accuracy of the GSA 1 samples. An overlapping was observed between samples from GSAs 6 and 12 another one between GSAs 3 and 4. These overlappings were due to errors in classification of samples from GSA 4 into GSA 3 and from GSA 6 into GSA 12. Two possible causes could explain misclassifications which are: (1) similarity of the environmental condition influencing otolith chemistry and or (2) mixing of individual composing one population. The otolith edge chemistry indicated relatively tighter clustering of the samples from GSAs 3 and 4 with classification accuracy corresponding to 66.7% and 63%. The separation of the GSA 3 samples from the other GSAs suggested that there is limited or no mixing of the adult hake from GSA 3 with the other hake among different areas. The same hypothesis could be stated for GSA 4 even though there is some overlapping with GSA 12 and GSA 6. The overlapping of the samples from GSA 12 with GSA 4 and of samples from GSA 1 with GSA 6 could be attributable to the wide spatial migration of the hake. Indeed, the overlap

of marginal otolith composition between these areas is probably indicative of similarity of the environment conditions influencing otolith chemistry.

The canonical variate plots of the otolith core chemistry involving samples from the different GSAs and North and south Atlantic indicated an overlapping of samples from GSA 3 with Atlantic samples. the otolith edge chemistry indicated relative tighter clustering of the samples from GSA 12 compared to the other GSAs. The overlapping observed between GSAs 1 and 6 and Atlantic north and south could be attributable to extensive migration of hake in these waters and so the fish had remained more closely associated in space and time resulting in individual having similar marginal otolith composition.

In conclusion, the results of the core otolith chemistry were not sufficiently different for consistent discrimination between fish from the different sampling. However, we can observe a tighter clustering of samples from GSA 4. GSA 12 clustered closer to GSA 6, which could be attributable to a one mixing population. GSA 3 clustered closer to Atlantic samples than to GSA 4.

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